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Distribution and In situ Survival and Activity of *Klebsiella pneumoniae* and *Escherichia coli* in a Tropical Rain Forest Watershed

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Abstract. For a period of 7 months water samples were analyzed for the presence of *Klebsiella pneumoniae* and fecal coliforms at 11 sites in a cloud rain forest watershed in Puerto Rico. Diffusion chamber studies were conducted at two sites which were found to contain low numbers of naturally occurring *K. pneumoniae* and fecal coliforms. These studies indicated that both *K. pneumoniae* and *Escherichia coli* may be capable of surviving environmental conditions for extended periods of time. The presence of both bacteria in pristine natural waters is indicative of their being autochtonous to tropical environments. Thus, as a result, the use of coliform and even fecal coliform bacteria as indicators of fecal pollution may be misleading when applied to countries with a tropical climate.

Plants and botanical byproducts have long been associated with increased densities of *Klebsiella pneumoniae* in the environment [6, 21, 32, 34]. High densities of *K. pneumoniae* have been demonstrated repeatedly in pulp and paper mills [8], textile mills [11], and in soil, bark, and water from temperate forests [12]. We have also found high densities of *K. pneumoniae* associated with run distillery effluents [4]. The ability of *K. pneumoniae* to fix nitrogen and its association with plants suggest an important environmental role for this bacterium [18, 20, 23].

Klebsiella pneumoniae is also an important pathogen of man and has been found to be the etiological agent of pneumonia [10, 28] and urinary tract infections [12, 28] and has been associated with scombroid food poisoning [30, 35]. Biochemical and serological studies of *K. pneumoniae* have demonstrated for the most part that no differences exist among human pathogenic strains and those obtained from the environment [22, 25]; however, this issue is still debated by some [2, 31].

Klebsiella pneumoniae as a fecal coliform-positive bacteria may represent as much as 70%–90% of the fecal coliform-positive isolates in waters associated with plant byproducts [15]. These data demonstrate that the presence of *K. pneumoniae* in the environment could interfere with the fecal coliform assay, thus overestimating the incidence of fecal pollution.

The waters in Puerto Rico are believed to be grossly contaminated by human waste. The US Water Resources Council reported that 96% of all sampling stations of 26 rivers in Puerto Rico demonstrated violations of the coliform standard [36]. However, the utility of applying methodology developed in temperate climates to measure fecal contamination in tropical climates is still in doubt. Other studies in tropical freshwater have shown that a high proportion of fecal coliform-positive isolates are of nonfecal origin [24]. In Puerto Rico, less than 30% of the fecal coliform-positive isolates from a variety of sites around the island are identified as *Escherichia coli* [15, 17] (Hazen, unpublished data). One such site is the tropical rain forest, where the upper parts of the watershed are known to have high fecal coliform counts in the absence of any identifiable fecal source [15]. The present study examines the distribution and in situ survival characteristics of *K. pneumoniae* and *E. coli* in the watershed associated with this tropical rain forest in Puerto Rico.

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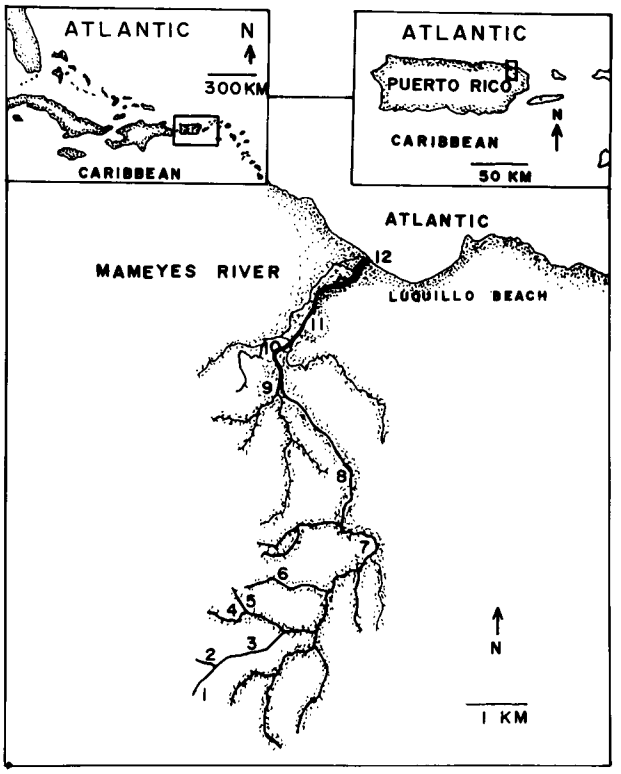

Fig. 1. Map showing location of study sites in the Mameyes River Watershed, Puerto Rico.

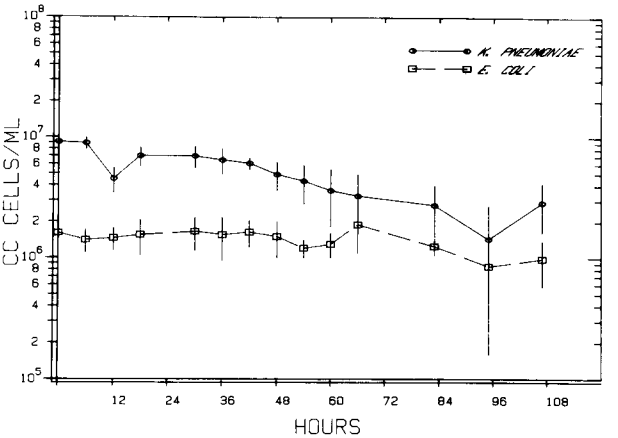

Fig. 2. Changes of in situ densities of *Klebsiella pneumoniae* and *Escherichia coli* by Coulter counter over time (mean ± 1 standard error).

Materials and Methods

Study site. The Mameyes River has a watershed with a drainage area of 30.6 km. The river begins at an elevation of nearly 1000 m above sea level. The upper third is in the Caribbean National Forest, a natural cloud rain forest, which receives about 762 cm of rain each year. The middle third receives primary treated domestic sewage from a large housing development. The lower third passes through two towns, where it is further polluted by domestic and industrial sewage effluents [15]. The river empties into the Atlantic Ocean near Luquillo Beach, Puerto Rico's largest public beach.

Bacteriological analyses. Water samples were placed in sterile Whirl-Pak bags (Nasco, Ft. Wilkinson, Wisconsin) and kept on ice until assayed. Samples were analyzed by the membrane filtration technique as indicated in [1], with use of m-FC for the detection of fecal coliforms. For the detection of *K. pneumoniae*, filters were placed on double violet red bile agar (DVA) and incubated at 37°C for 24 h. Any glistening, lavender colonies were counted as presumptive *K. pneumoniae* [7]. Random isolates were further characterized as *K. pneumoniae* with API-20E strips (Analytab Products, Plainview, New York).

Survival studies. A modification of the McFeters and Stuart method [26] was used to study survival characteristics of *K. pneumoniae* and *E. coli* at two sites (sites 4 and 5, Fig. 1) found to contain relatively low numbers (when compared with other sampling sites) of indigenous *K. pneumoniae* and fecal coliforms. Nylon-reinforced Versapor membrane filters (0.45-μm pore size) (Gelman Instrument, Ann Arbor, Michigan) were loaded onto diffusion chambers with 100-ml volume capacity. Pure cultures of *K. pneumoniae* and *E. coli* were grown in 5% tryptic soy broth for 24 h. Cells were harvested by high-speed centrifugation and resuspended in filter-sterilized, phosphate-buffered saline (pH 7.0). Number of cells was determined with a model ZF (Coulter Electronics, Hialeah, Florida) and the concentration adjusted to 1×10^7 cells ml^{-1}. Suspensions were placed into sterile diffusion chambers just prior to their being placed at the study sites. Five replicate chambers were used for each bacterium. The chambers were placed at a depth of 0.5 m. Periodically, 1-ml samples were obtained from each chamber. Of each sample, 0.5 ml was fixed with 1.5 ml of phosphate-buffered formalin for later counting at the laboratory with use of a Coulter Counter as described by Hazen and Esch [16]. Another 0.5 ml was subjected to a modified technique of Zimmerman et al. [38]. The sample was mixed with 0.5 ml of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), incubated in the dark for 20 min, and fixed with 0.01 ml of formaldehyde (37% vol/vol in water). The preserved sample was then stored on ice for transport to the laboratory. The percentage of bacteria respiring was determined by counting those cells containing dark granules of INT–formazan (Fig. 4). Direct counts and activity measurements were done on the same sample by staining with acridine orange (AODC, 1 : 10,000) and counting the number of red (active) and green (nonactive) fluorescing cells after filtration through an Irgalan black-stained, 0.2-μm pore size polycarbonate membrane (Nucleopore, Pleasantville, California) [19] (Fig. 3). All statistics according to Zar [37].

Results

During the sampling period, the physicochemical parameters measured showed great variability within the same sites. Generally, higher measurements for alkalinity, sulfate, phosphate, and total

Table 1. Mameyes River watershed physicochemical and bacteriological quality[a]

Site	Temp (°C)	Dissolved oxygen (mg/l)	Alkalinity (mg $CaCO_3$/l)	Nitrites + nitrates (mg/l)	PO_4^{-2} (mg/l)	Total phosphorus (mg/l)	Sulfates (mg/l)	Chlor. A trichromate (mg/l)	Fecal coliforms (CFU/10 ml)	*Klebsiella pneumoniae* (CFU/100 ml)
1	21 ± 0.5	7.1 ± 0.9	20 ± 0	0.35 ± 0.35	0.89 ± 0.31	2.9 ± 1.1	6.12 ± 0.12	5.7 ± 5.0	53.6 ± 16.6	2.4 ± 2
2	20 ± 0.5	6.9 ± 1.1	30 ± 10	0.39 ± 0.39	0.89 ± 0.31	2.8 ± 1.8	5.5 ± 0.42	22.4 ± 21	40 ± 58	2.3 ± 1.8
3	20 ± 0.5	7.8 ± 0.45	20 ± 0	0.44 ± 0.44	1.47 ± 0.48	4.82 ± 0.5	6.5 ± 1.3	8.6 ± 7.5	130 ± 180	3.75 ± 5
4	21 ± 0.5	7.7 ± 0.5	40 ± 0	0.02 ± 0.02	1.29 ± 0.1	3.3 ± 1.7	2.5 ± 2.5	11.1 ± 10	5 ± 5	4.25 ± 2.5
5	21 ± 0.5	7.9 ± 0.75	50 ± 20	0.19 ± 0.05	1.14 ± 0	2.9 ± 0.1	6.7 ± 3.4	5.5 ± 5.3	32 ± 39	21 ± 39
6	21 ± 0.25	7.5 ± 0.7	30 ± 10	0.14 ± 0.07	2.31 ± 0.71	6.9 ± 6.0	7.9 ± 0	18.9 ± 18.7	11 ± 9.3	10.8 ± 12
7	23 ± 0.5	7.3 ± 1.2	30 ± 10	0.44 ± 0.44	6.73 ± 3.5	5.3 ± 3.8	7.6 ± 1.6	30 ± 29	69 ± 62	9.5 ± 10
8	24 ± 0	7.2 ± 0.6	50 ± 20	0.45 ± 0.43	2.98 ± 0.83	2.9 ± 0.6	6.9 ± 0.9	62.6 ± 60.6	10.4 ± 131	26 ± 37
9	25 ± 0.5	6.6 ± 1.0	50 ± 20	0.28 ± 0.07	6.28 ± 2.1	8.34 ± 2.1	11.2 ± 1.9	41.6 ± 39	2,196 ± 2,748	31 ± 20
10	25 ± 0.25	7.4 ± 1.0	40 ± 0	0.12 ± 0.04	4.77 ± 0.85	10.2 ± 1.4	8.82 ± 0.8	44 ± 42	107 ± 60	6.3 ± 8.2
12	25 ± 0.25	6.2 ± 1.0	130 ± 70	0.17 ± 0.12	3.56 ± 1.9	8.6 ± 3.9	81.7 ± 1.3	54 ± 53	134 ± 188	22 ± 40

[a] All numbers are average values of all measurements taken during the study ± standard deviation.

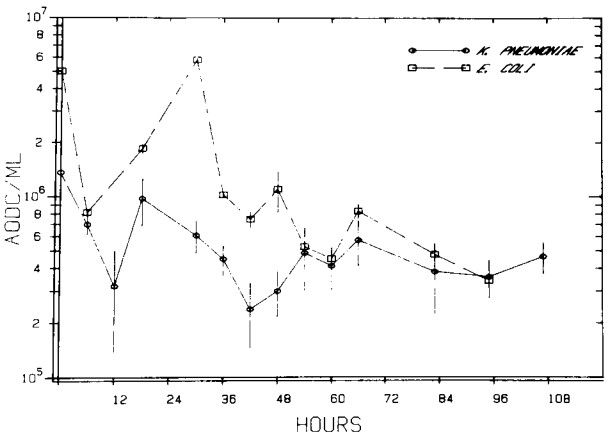

Fig. 3. Changes of in situ densities of *Klebsiella pneumoniae* and *Escherichia coli* by AODC over time (mean ± 1 standard error).

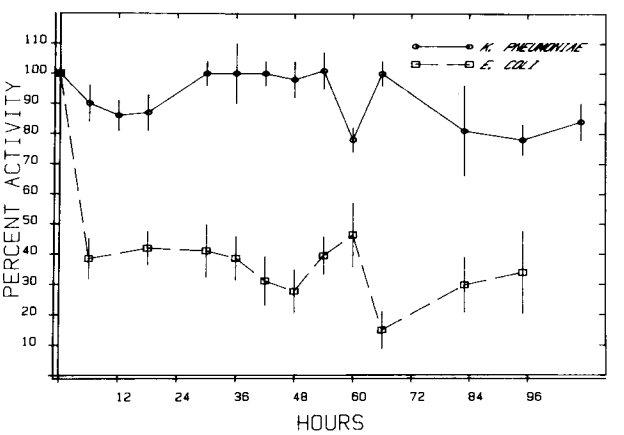

Fig. 4. Changes of in situ activity of *Klebsiella pneumoniae* and *Escherichia coli* by AODC over time (mean ± 1 standard error).

phosphorus were obtained from the lower parts of the watershed (Table 1). Torrential rain fall (>10 cm h^{-1}) creates rapidly changing turbidity; thus, turbidity was not significantly different by site.

Densities of fecal coliforms were higher at sites 3, 9, and 12 (Table 1). Average concentrations in all sampling sites ranged from 5 ± 5 colony-forming units (CFU)/100 ml to 2196 ± 2748 CFU/100 ml (Table 1). *Klebsiella pneumoniae* was isolated from all sites samples. Concentrations ranged from 2.0 CFU/100 ml to 52 CFU/100 ml. In all cases the standard deviations were as large as or even larger than the average value.

Survival measurements of *K. pneumoniae* revealed only a 66% decrease in the number of cells even after 108 h of exposure to environmental conditions as determined by Coulter Counter counts (CC) (Fig. 2). *Escherichia coli* cells showed only a 33% decrease over the same time period (Fig. 2). On the other hand, acridine orange direct counts (AODC) showed slightly different results. Data shown in Fig. 3 indicate a 67% and a 80% decrease after only 36 h for *K. pneumoniae* and *E. coli*, respectively. The levels of *K. pneumoniae* remained constant for the rest of the sampling period. *Escherichia coli* cell concentration continued falling until a 90% decrease was observed after 108 h.

When looking at the percentage of activity with AODC, 90% of the *K. pneumoniae* cells remained active for the whole sampling period (Fig. 4). After a period of 6 h, only 40% of the *E. coli* cells were active. The percentage of activity of *E. coli* dropped slightly after this initial drop and >30% of the cells remained active after 96 h.

The original percentage of respiring *K. pneumoniae* cells was observed to be low (only 25%), but

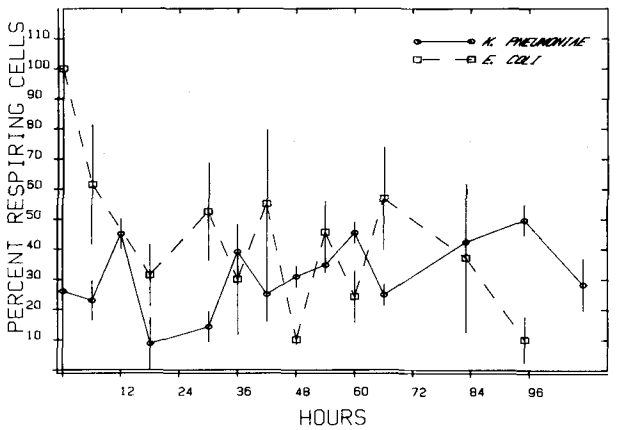

Fig. 5. Changes of in situ respiring cell percentages of *Klebsiella pneumoniae* and *Escherichia coli* by INT over time (mean ± 1 standard error).

remained constant during the whole sampling period (Fig. 5). *Escherichia coli,* on the other hand, had a high percentage of cells respiring (100%) at the beginning of the sampling period, but the percentage dropped to 30 after only 18 h. At the end of 96 h, only 10% of the *E. coli* cells were observed to be respiring.

Discussion

Water quality in the Mameyes river watershed seems to be controlled, at least partially, by the torrential rainfall. These torrential rainfalls purge the river of most nutrients and scour the streams of debris and sediment. Only at sampling stations below site 7 was any appreciable sediment buildup observed. Similar observations have been made in more detailed studies of the watershed [14] (C.F. Aranda, MS thesis, University of Puerto Rico, Rio Piedras, 1982). Thus, turbidity could go from 100% transmittance to 0% and back to 100% before and after rainfall. No seasonal fluctuations were observed in the physicochemical or biological water quality parameters measured, and any fluctuations that did occur seemed to be caused mainly by increases in precipitation (data not shown). Water temperature at all sites varied less than 2°C during the entire sampling period. The upper parts of the watershed were relatively nutrient poor as evidenced by low values for phosphates, nitrates, and chlorophyll A. In contrast, the lower parts of the watershed increased in nutrient concentrations. Much of this increase in nutrients seemed to be a result of the sewage discharge into the river at site 9. However, relatively high concentrations of phosphates, nitrates, and chlorophyll A were observed as high up as site 7.

Fecal coliform densities were highest at and below the sewage point source (site 9), even though fecal coliforms were detected in all sites sampled. The concentrations of fecal coliforms isolated were lower than those reported by Evison and James for river samples taken in two countries in tropical Africa and much higher than those reported from river samples taken in England [13]. One site downstream from the sewage point source violated the currently accepted standard for fecal coliforms in recreational waters (i.e., >200 CFU/100 ml). If one takes into consideration the standard deviations, four sites (3, 8, 9, and 12) violated these standards at some time during the study. Concentrations of total coliforms (data not shown) were also found to exceed federal standards for recreational waters (i.e., <1000 CFU/100 ml). Statistical analyses indicated a positive correlation between concentrations of coliforms and increasing concentrations of phosphates, total phosphorus, and nitrates (data not shown). Thus, fecal coliforms follow a familiar pattern of being associated with productive environments and high nutrients, conditions that would invite regrowth [5, 13, 14, 27]. The large standard deviations shown in Table 1 indicate that in many cases no bacteria were isolated from the sites. This may be indicative of the presence of *K. pneumoniae* and fecal coliforms in the sediment or epilithic communities that may be stirred up after heavy rainfall. Rainstorms may also flush these bacteria from luxuriant vegetation in the watershed (we are currently investigating these hypotheses in our laboratory).

Klebsiella pneumoniae cells were isolated from all sites sampled, and the concentrations did not seem to correlate with any of the parameters measured. The highest average concentration was observed at site 9. However, the concentrations isolated did not seem to be dependent on site. The densities of *K. pneumoniae* reported in this study are lower than those for pulpmill effluents (<1000 CFU ml^{-1}) [8, 22, 29], but higher than those reported for uncontaminated waters (<0.1 CFU ml^{-1}) [3, 8, 22].

Bacterial survival experiments with pure cultures of *E. coli* and *K. pneumoniae* in diffusion chambers gave slightly varying results depending on the method used for measuring the number of cells. When using CC counts, *K. pneumoniae* cells

seemed to decrease in number much faster than *E. coli* cells over time. When measurements of cell densities were done by the AODC method, we observed that *E. coli* numbers decreased much faster than did those of *K. pneumoniae*. However, both sets of data indicate that a significant percentage of cells still survived up to 108 h of being exposed to environmental conditions. In fact, 33% of the *K. pneumoniae* cells were found to survive for up to 108 h, while >66% of *E. coli* cells survived for the same time period (when measurements were done using CC counts).

Acridine orange direct counts indicated >33% of *K. pneumoniae* and 20% of *E. coli* cells survived for the same period as above. The difference in values observed when using different methods is possibly a result of the intrinsic idiosincracies in each method. The Coulter counter may measure nonviable cells, or even noncellular material, and thus result in an overestimation in the number of cells present. The fluorescence of cells in the AODC method is a result of the attachment of the dye to molecules of DNA and RNA [9]; therefore, only cells should be visible when observed under epifluorescent microscopy. However, background fluorescence may also interfere with an exact bacterial count. Nonetheless, in spite of these differences in results, both methods indicate survival of both *K. pneumoniae* and *E. coli* under environmental conditions.

The percentage of activity as measured by the ratio of green- versus red-fluorescing cells seems to be dependent on the amount of RNA present within the cells. Acridine orange fluoresces red when bound to single-stranded nucleic acids [9], and thus an increase in activity would be demonstrated as an increase in mRNA, which in turn would result in red-fluorescing cells. Using this hypothesis, we measured the "activity" of *K. pneumoniae* and *E. coli* in diffusion chambers. The percentage of activity at the beginning of the experiment was found to be 100%; 90% of the *K. pneumoniae* cells remained active throughout the whole sampling period of 108 h. The percentage of activity of *E. coli* cells decreased to <40% within 6 h; however, this 40% activity remained relatively constant throughout the rest of the sampling period (up to 96 h).

The percentage of respiring cells was measured by the ability of cells to reduce INT to INT–formazan (INT serves as a synthetic electron acceptor and is, therefore, reduced) [38]. The number of cells containing dark granules were presumed to be respiring. The difference in physiological activity between *K. pneumoniae* and *E. coli* was found to be much more prevalent with INT reduction than with AODC. The original percentage of respiring *K. pneumoniae* cells was found to be only 25%. This low percentage was constant throughout the whole sampling period. *Escherichia coli* cells indicated an enormous decrease in the percentage of respiring cells after only 18 h (even though 100% of the cells were observed to be respiring at time zero). At the conclusion of the sampling period, only 10% of the cells were observed to be respiring. Even with the differences encountered as a result of use of two different methods for the determination of survival of *K. pneumoniae* and *E. coli* in the environment, it is obvious that both bacterial types are capable of survival.

Both of these sets of data indicate that *K. pneumoniae* and *E. coli* can survive and remain physiologically active under the environmental conditions to which they were exposed. Sjorgren and Gibson [33] indicated that *K. pneumoniae* is able to survive at a rate of >58% for up to 5 days in lake water at in situ temperature. The present study confirms and expands the notion that *K. pneumoniae* is a "survivor" in the environment, but in addition the study shows that these bacteria are physiologically active. This study also indicates that *E. coli* can survive for long periods of time in the environment. Thus, the physiological activity retained by these bacteria not only indicates survival, but also indicates the possibility of their becoming part of the normal flora in tropical fresh-water environments. The latter point is further corroborated by the isolation of these two genera of bacteria from tropical rain forest environments without any source of fecal pollution. Our laboratory routinely isolates fecal coliforms from tropical environments. Thus the data presented here indicate that fecal coliforms would seem to be poor indicators of recent fecal contamination. These findings raise further doubts as to the efficacy of applying fecal coliform bacteria as indicators of water quality in tropical fresh-waters. This study also suggests that potential pathogens (such as *K. pneumoniae* and certain strains of *E. coli*) will remain viable for long periods of time in tropical environments. Further studies are necessary to determine whether pathogenicity of these bacteria remains unaltered.

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